Effect of Long-Term Excessive L-Methionine Consumption on Transferrin Receptor Abundance and Mitochondrial H_2O_2 Generation in Rat Liver

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Iron acquisition is a fundamental requirement for many aspects of life, but excess iron may result in the formation of free radicals that damage cellular constituents. Therefore, the amount of iron within the cell is carefully regulated by the iron metabolism (IRE/IRP system) in order to assure an adequate level. We previously reported that long-term excessive L-methionine consumption increases iron and lipid peroxide levels in rat liver. To determine whether such excess iron accumulation depends on the elevation of transferrin receptor *via* oxidative stress, we investigated the possible effects of long-term excessive L-methionine intake on the iron metabolism and the mitochondrial function in rat liver. Wistar male rats were fed either an L-methionine-supplemented (16.0 g/kg) diet or a control diet for 3 and 6 mo. The expression of transferrin receptor is significantly elevated by excess L-methionine intake, indicating that an accumulation of iron may be accompanied by such an elevation in the liver. Long-term excessive L-methionine consumption significantly decreases the H_2O_2 production of rat liver mitochondria without inducing changes in mitochondrial oxygen consumption. No significant differences in either glutathione peroxidase activity or superoxide dismutase activity were shown between the two groups. In contrast, the glutathione concentration significantly increased in L-methionine-treated rats compared to controls. These results indicate that long-term consumption of excess L-methionine by rats may affect mitochondrial function, resulting in a reduction in H_2O_2 generation. Moreover, an accumulation of iron by excess L-methionine intake may be responsible for a mechanism other than the IRE/IRP system *via* mitochondrial oxidative stress.

Key words —— methionine, transferrin receptor, H_2O_2 generation, iron metabolism, mitochondria

INTRODUCTION

As an essential constituent of many cellular macromolecules, iron participates in numerous biochemical activities, such as oxygen transport and electron transfer reactions.[1] An excess of iron is, however, toxic to cells, and the pathologic conditions of iron overload are associated with tissue injury and degeneration. The reactivity of ferrous/ferric ions against hydrogen peroxide (H_2O_2) and superoxide (O_2^-) to yield hydroxyl radicals in the Fenton/Haber-Weiss reactions provides a molecular basis for iron toxicity.[2] Considering that reactive oxygen species (ROS), including H_2O_2 and O_2^-, are inevitable byproducts of aerobic respiration, cells must strictly control iron homeostasis to minimize the toxic effects of iron.

The key proteins in iron metabolism are transferrin, transferrin receptor (TfR) and ferritin. The expression of transferrin is mainly regulated transcriptionally. On the other hand, those of TfR and ferritin are co-translationally controlled by intracellular iron levels *via* specific mRNA-protein interactions in cytoplasma.[3–5] Particular hairpin structures, called iron-responsive elements (IRE) in the respective mRNAs, are recognized by *trans*-acting proteins known as iron-regulatory proteins (IRPs), and this controls the rate of mRNA translation or stability. Recently, it was revealed that signals other than iron levels could regulate IRPs and thus modulate cellular iron levels. IRP (especially IRP-1) responds to additional, iron-independent signals such as NO and oxidative stress.[4,6,7] Thus, the physiology of oxidative stress is very closely connected with regulation of the iron metabolism.

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ROS are generated from mitochondria, the intracellular organelles that produce adenosine triphosphate (ATP), and constitute the greatest source of steady-state oxidants. The mitochondrial electron transport system consumes more than 85% of all the oxygen used by the cells, and it is estimated that between 1 and 5% of the oxygen consumed by mitochondria is converted to O_2^- at intermediate steps of the mitochondrial respiratory chain. That O_2^- is then readily dismutated by mitochondrial superoxide dismutase (MnSOD), leading to the production of H_2O_2. Under physiological conditions, moreover, H_2O_2 is detoxified by glutathione peroxidase (GPX) or intra-and extra-mitochondrial catalase.[8–10] Due to conditions under which O_2^- generation increased or the antioxidant system in mitochondria is depleted, H_2O_2 may accumulate, causing mitochondria to be in an oxidative stress condition.

We previously investigated the effects of long-term excessive L-methionine consumption on the metabolism of iron and antioxidants in the liver.[11] Our results in that study suggested that excess L-methioine intake may induce oxidative stress in the liver, disrupting the iron metabolism *via* the IRE/IRP system, thereby resulting in the accumulation of iron in the liver. This led us to speculate that excess L-methionine consumption affects mitochondrial function and increases the H_2O_2 generation of rat liver mitochondria, which may then induce an elevation of TfR expression, resulting in an accumulation of iron in the liver. The objective of the present study was to investigate the possible effects of excessive L-methionine consumption on hepatic TfR expression, oxygen consumption, H_2O_2 generation and the antioxidant defense system in rat liver mitochondria.

MATERIALS AND METHODS

Animals and Treatment —— Male Wistar rats (7 weeks old, 250–270 g) were purchased from Clea Japan (Tokyo, Japan). The rats were divided into groups of 3 or 4 and housed in hanging stainless steel wire cages with free access to a standard diet (CE2; Clea Japan) and water in a room with controlled temperature (22 ± 1–2°C). Lights were maintained on a 12 hr light : dark cycle (lights on from 7:00 to 19:00). After being accilimated to the facility for 5 days, they were divided on day 6 into 2 groups (n = 6 or 7/group) as follows: a control group fed control diets and a methionine group receiving L-methionine-supplemented diets (MSD). Both groups were given free access to their respective experimental diets and tap water. Body weight and food intakes were recorded throughout this study. Care and treatment of the animals conformed to the requirements of the Center for Animal Resources and Development, Kumamoto University.

Table 1. Composition of Experimental Diet[a)]

Ingredient	Control Diet	MSD
	g/kg	
Casein	140	140
Cornstarch	466	450
Dextrinized Cornstarch	155	155
Sucrose	100	100
Cellulose	50	50
Soybean-Oil	40	40
Mineral Mixture[b)]	35	35
Vitamin Mixture[c)]	10	10
L-Cystine	1.8	1.8
L-Methionine	—	1.6
Choline Bitartrate	2.5	2.5
t-Butylhydroquinone[d)]	0.008	0.008

a) Based on AIN-93M Rodent diets.[12] *b*) Mineral mix of AIN-93M-MX per kg diet: $CaCO_3$, 12.5 g; KH_2PO_4, 8.75 g; NaCl, 2.59 g; K_2SO_4, 1.631 g; $C_6H_5K_3O_7 \cdot H_2O$, 0.98 g; MgO, 0.84 g; $FeC_6H_6O_7 \cdot nH_2O$, 0.21 g; $ZnCO_3$, 58 mg; $MnCO_3$, 22 mg; $CuCO_3$, 11 mg; KIO_3, 0.35 mg; Na_2SeO_4, 0.36 mg; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.28 mg; $Na_2SiO_3 \cdot 9H_2O$, 50.8 mg; $CrK(SO_4)_2 \cdot 12H_2O$, 9.63 mg, H_3BO_3, 2.85 mg; NaF, 2.22 mg; $NiCO_3$, 1.11 mg; LiCl, 0.61 mg; NH_4VO_3, 0.23 mg. *c*) Vitamin mix of AIN-93-VX per kg diet: nicotinic acid, 30 mg; Ca pantothenate, 16 mg; pyridoxine-HCl, 7 mg; thiamin-HCl, 6 mg; riboflavin, 6 mg; folic acid, 2 mg; D-biotin, 0.2 mg; cyanocobalamine, 25 mg; all-*rac*-α-tocopheryl acetate, 150 mg; all-*trans*-retinyl palmitate, 8 mg; cholecalciferol, 2.5 mg; phylloquinone, 0.75 mg. *d*) Added as an antioxidant.

The compositions of the experimental diets (control diets and MSD) are shown in Table 1. The control diet was based on the AIN Rodent Diets, AIN-93M, containing 14% casein, and was obtained from Dyets (Bethlehem, PA, U.S.A.). Reeves *et al*. proposed that for long-term studies using nonpregnant animals and after completion of the rapid growth phase, they should be provided with the AIN-93M diet that we adopted for our study.[12]

After 3- and 6-months of consuming the experimental diets, the rats were anesthetized with diethyl ether, and blood was collected from the heart by injection syringe and put into plastic tubs containing a serum-separating reagent (Venoject II, Terumo, Tokyo, Japan). The serum was separated from whole blood by centrifugation at 800 × g for 10 min. The rats were then perfused with ice-cold saline, and the liver was quickly removed. In addition, an aliquot

of liver was immediately used for mitochondria preparation, and the remainder was cut into several portions, weighted again, immediately frozen using dry-ice/isopropylalcohol, and stored at –80°C until analysis for several components.

Determination of Iron —— Liver samples were homogenized in 4 volumes of an ice-cold 50 mM potassium phosphate buffer, pH 7.0, 1 mM (±)-dithiothreitol, 5 mM 4-(2-aminoethyl)-benzensulfony fluoride hydrochloride and 1% BHT-ethanol. An aliquot of this liquid was heated at 80°C for 15 min and centrifuged at 1000 × *g* for 15 min at 4°C and the supernatants thus obtained were used as the ferritin fraction.

Hepatic iron concentration was determined by flame atomic absorption spectrophotometry. Serum iron and total iron-binding capacity (TIBC) were measured with Fe C-Test and UIBC-Test kits, respectively (Wako Pure Chemical, Osaka, Japan).

Hepatic Transferrin Receptor Determination —— Subcellular fractionation for the measurement of TfR was conducted according to Sciot *et al*.[13] The livers were minced and homogenized in an ice-cold 0.25 M sucrose using a loose-fitting glass-teflon homogenizer with 8 strokes. The homogenate was centrifuged at 10000 × *g* for 10 min. The resulting supernatant was saved, and the pellet gently resuspended and centrifuged again at 10000 × *g* for 10 min. The supernatant from the first and second centrifugations were pooled and centrifuged at 100000 × *g* for 90 min. The obtained pellet was resuspended in 0.25 M sucrose as the enriched membrane fraction (EMF), which is enriched in golgi body, endoplasmic reticulum, endosomal and plasma membranes, and excludes mitochondrial and nuclear membranes.

The TfR concentration was determined by ELISA using two types of antibodies against rat TfR.

Isolation of Mitochondria from Liver —— Liver mitochondria were isolated according to the method of Lash and Sall.[14] Fresh liver was minced with a scissors and homogenized in 10 volumes of isolation buffer, which consisted of 10 mM potassium phosphate buffer, pH 7.4, 225 mM sucrose, 5 mM $MgCl_2$, 20 mM KCl, 20 mM triethanolamine-HCl, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), and 2 mM EGTA, using a Dounce homogenizer. The homogenate was centrifuged at 600 × *g* for 10 min. The resulting pellet was resuspended in a 1/2 initial volume of the buffer using a Dounce homogenizer and again centrifuged at 600 × *g* for 10 min. The combined supernatants were centrifuged at 15000 × *g* for 5 min. The resulting pellet was carefully rinsed and resuspended in the isolation buffer without EGTA. Protein concentration was determined according to the method of Lowry *et al*. using bovine serum albumin as a standard.[15]

Oxygen Consumption by Isolated Mitochondria —— Oxygen consumption was measured in freshly isolated mitochondria using a Clark-type electrode and a YSI Model 5300 biological oxygen monitor (YSI Inc., Yellow Springs, OH, U.S.A.) at 28°C. Polarographic recordings were continuously displayed on a chart recorder (Model FBR-252A, TOA Electronics Ltd., Tokyo, Japan). For calibration, the oxygen content of an air-saturated respiration buffer was assumed to be 227 nmol O_2/ml under the conditions of the experiment. The respiration buffer contained 10 mM Tris–HCl, pH 7.4, 100 mM KCl, 75 mM mannitol, 25 mM sucrose, 5 mM phosphate, 0.05 mM EDTA (K^+ salt). Respiratory substrates were added to the aliquots of mitochondrial protein (1.5–3.0 mg) in the following final concentrations: 2.5 mM malate, 2 mM glutamate, 10 mM succinate, 0.5 mM adenosine diphosphate (ADP). The conditions of incubation for state 4 respiration (basal metabolism of substrate), state 3 respiration (ADP-stimulated metabolism of substrate), and the respiratory control ratio (RCR) were as defined by Estabrook.[16]

Measurement of Mitochondrial H_2O_2 —— H_2O_2 release from isolated mitochondria was assayed using a modification of the method of Ruch *et al*.[17] Fresh isolated mitochondria of a known protein concentration (200 μg) were preincubated at 28°C for 10 min in the presence of the respiratory substrate (same final concentrations as above), and were then incubated at 28°C for 30 min in the presence of the respiration buffer, horseradish peroxide (2 U/ml), and 100 nmol of homovanillic acid (4-hydroxyl-3-methoxy-phenylacetic acid). The reaction was stopped with 0.1M glycine-NaOH buffer, pH 12, containing 25 mM EDTA (K^+ salt), and the samples were then centrifuged at 1900 × *g* for 10 min. Fluorescence was detected by a fluorospectrophotometer (F-2000, Hitachi, Japan) at an excitation wavelength of 321 nm and an emission wavelength of 425 nm. The exact H_2O_2 concentration of the solution used to establish each standard curve was determined spectrophotometrically at 230 nm, assuming an extinction coefficient of 61 $M^{-1}cm^{-1}$. Mitochondrial H_2O_2 generation and oxygen consumption were measured in parallel in the same sample under the same experimental conditions.

Determination of Mitochondrial Antioxidants ——— Mitochondria were disrupted by detergent treatment as described by Lash and Sall.[14] Freshly isolated mitochondria were gently mixed with equal volume of ice-cold digitonin (0.11 mg/mg protein) by stirring for 15 min, then diluted with 3 volumes of fractionation buffer [2 mM HEPES-KOH, pH 7.4, 220 mM mannitol, 70 mM sucrose, 0.5 mg/ml bovine serum albmin (BSA) (fatty acid free)], and gently homogenized with a Dounce homogenizer. The homogenate was centrifuged at $9500 \times g$ for 10 min. The obtained supernatant was discarded and the pellet was resuspended in the initial volume of digitonin buffer using a Dounce homogenizer. The homogenate was again centrifuged at $9500 \times g$ for 10 min, and the resulting pellet (mitoplasts) was resuspended in 1/3 the initial volume of fractionation buffer, and treated with Lubrol PX (0.1 mg/mg mitoplasts protein). They were then gently homogenized and centrifuged at $144000 \times g$ at 4°C for 60 min. The resulting supernatants were used. Total and selenium GPX (T-GPX and Se-GPX) activities and superoxide dismutase (SOD) activities were measured according to the method of Lawrence and Bürk[18] and Spitz and Oberley,[19] respectively. Glutathione concentration was measured with a Total Glutathione Quantification Kit (Dojindo Molecular technologies, Inc., Kumamoto, Japan).

Statistical Analysis ——— Data are expressed as means ± S.D. The significance of difference between the two groups at each feeding period was analyzed statistically by Welch's *t*-test using Statcel software (OMS, Saitama, Japan). Differences were considered significant at $p < 0.05$.

RESULTS

Growth and Food Intakes

At the end of the feeding periods (3 and 6 months), body weights of the rats fed MSD were significantly lower than those of rats fed the control diet (Fig. 1). Voluntary food intake in rats fed MSD tended to be lower at the early stages (0–2 months) but not at the later stages (2–6 months) (data not shown).

Iron Concentration in Liver and Serum

Hepatic iron concentrations were significantly higher in MSD-fed rats than in controls at 3 and 6 months (Fig. 2). In particular, iron levels of the non-ferritin fraction in MSD-fed rats were much higher than controls at 6 months. On the other hand, serum TIBC levels were significantly lower in MSD-fed rats than in controls, while serum iron levels showed no significant difference between both groups (Fig. 3).

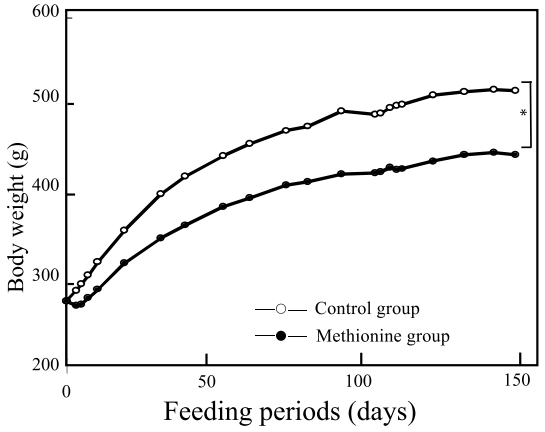

Fig. 1. Body Weights of Rats Fed Control or MSD

Significant difference ($p < 0.05$) between the groups at the end of feeding. ○: control group. ●: methionine group.

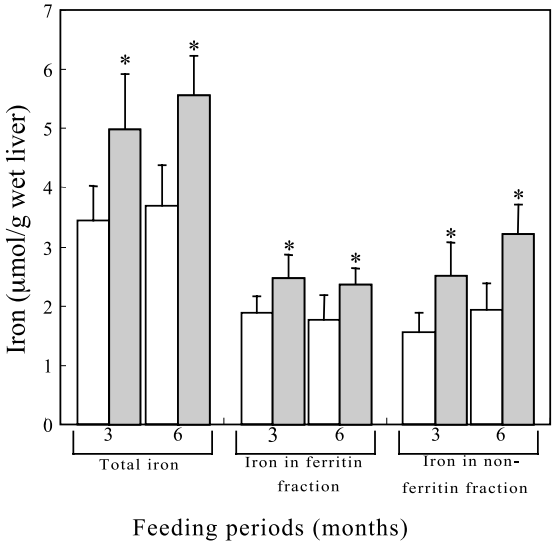

Fig. 2. Hepatic Iron Levels in Rat Fed Control or MSD

Mean ± S.D. of 5 or 6 animals per group. *Significant difference ($p < 0.05$) from control group at the end of each feeding period. □: control group. ■: methionine group.

Hepatic Transferrin Receptor Levels

Hepatic TfR levels were significantly higher in MSD-fed animals than controls at 6 months, whereas they did not differ between the two groups at 3 months (Fig. 4).

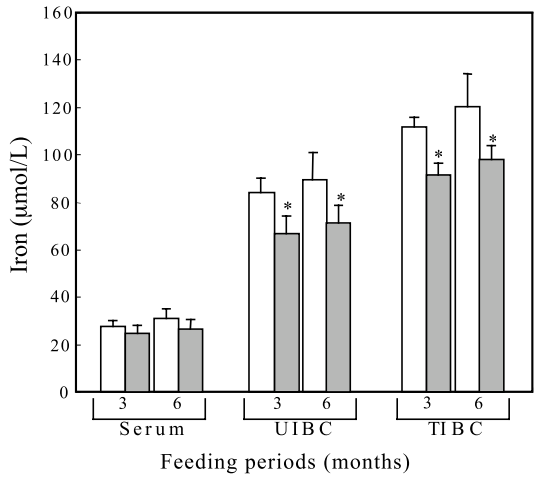

Fig. 3. Serum Iron Levels in Rat Fed Control or MSD

Mean ± S.D. of 5 or 6 animals per group. *Significant difference ($p < 0.05$) from control group at the end of each feeding period. □: control group. ■: methionine group.

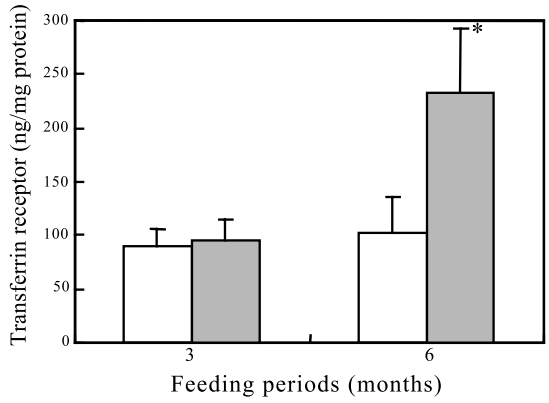

Fig. 4. Effect of Methionine Consumption on Transferrin Receptor Abundance in Rat Liver

Mean ± S.D. of 5 or 6 animals per group. *Significant difference ($p < 0.05$) from control group at the end of each feeding period. □: control group. ■: methionine group.

Mitochondrial Oxygen Consumption

Oxygen consumption in liver mitochondria from control and MSD-fed rats were shown in Table 2. Mitochondrial oxygen consumption did not differ between control and MSD-fed rats either in state 4 or state 3 respirations with any substrate at 3 and 6 months.

Mitochondrial Hydrogen Peroxide Generation

At 3 months, the rate of H_2O_2 generation with malate/glutamate and ADP (state 3) was significantly lower in MSD-fed rats than in controls (Table 3). Similarly, in the absence of ADP (state 4), H_2O_2 generation was lower in MSD-fed liver mitochondria than in control ones (38% reduction, although not significant). However, no significant difference was observed between control and MSD-fed animals with succcinate as substrate in both respirations.

At 6 months, the rate of H_2O_2 generation with malate/glutamate (state 4) significantly decreased by MSD feeding. Similarly, in the presence of ADP (state 3), H_2O_2 generation was lower in MSD-fed mitochondria than in control ones (39% reduction, although not significant). Moreover, MSD feeding significantly decreased H_2O_2 generation rate with succinate as substrate in both state 3 and state 4 respiration.

Antioxidant Enzyme Activities in Mitochondria

T-GPX, Se-GPX and SOD activities were not affected by MSD-feeding at any time, except for glutathione concentration, which was higher in MSD-fed rats than controls at 6 months (Table 4).

DISCUSSION

This investigation showed that long-term excessive L-methionine consumption elevated iron levels in rat liver, which were accompanied by enhancement of TfR expression in the liver. On the other hand, MSD feeding decreased the H_2O_2 generation rate of rat liver mitochondria, though it did not alter their oxygen consumption.

Iron is essential for numerous physiologic functions, from oxygen transport and utilization to DNA synthesis and xenobiotic detoxification, but excess iron may result in the formation of free radicals that damage cellular constituents. Therefore, it is of utmost importance for both the cells and the organism as a whole to maintain iron homeostasis to ensure a sufficient supply of iron while preventing its accumulation to dangerous levels. In vertebrates, iron uptake by the TfR as well as intracellular iron storage in ferritin are controlled by intracellular iron *via* IRE-IRP interactions.[3–5]

Furthermore, it was revealed that signals other than iron levels could regulate IRP and modulate cellular iron metabolism.[4,7] Binding capacity of IRP to IRE is also affected by nitric oxide,[6,20,21] phosphorylation by protein kinase C, hypozia/reoxigeneration[22] and changes in cell proliferation or differentiation. Moreover, its capacity is activated rapidly by oxidative stress, leading to increased TfR mRNA levels and suppression of ferritin synthesis.[6,23–25] In our study, MSD feeding increased he-

Table 2. Mitochondrial Oxygen Consumption in Rats Fed Control (C) and MSD

	3 mo		6 mo	
Substrate	C ($n = 5$)	MSD ($n = 5$)	C ($n = 6$)	MSD ($n = 6$)
Succinate				
State 4	10.36 ± 1.40	11.84 ± 1.41	10.74 ± 2.12	9.98 ± 1.06
State 3	50.98 ± 3.37	49.30 ± 8.84	49.05 ± 7.51	46.13 ± 6.33
RCR	4.97 ± 0.58	4.16 ± 0.54	4.65 ± 0.70	4.64 ± 0.59
Malate+glutamate				
State 4	6.40 ± 1.34	7.49 ± 1.15	6.02 ± 1.23	5.93 ± 1.29
State 3	29.62 ± 2.66	29.26 ± 4.59	29.15 ± 4.51	30.11 ± 4.16
RCR	4.75 ± 0.74	3.94 ± 0.55	4.98 ± 1.12	5.17 ± 0.68

Results, except for RCR, are expressed as nmol oxygen consumed per mg protein.

Table 3. Mitochondrial Hydrogen Peroxide Generationin Rats Fed Control (C) and MSD

	3 mo		6 mo	
Substrate	C ($n = 5$)	MSD ($n = 5$)	C ($n = 6$)	MSD ($n = 6$)
Succinate				
State 3	0.350 ± 0.148	0.305 ± 0.099	0.148 ± 0.029	0.092 ± 0.018[a)]
State 4	0.345 ± 0.094	0.279 ± 0.089	0.135 ± 0.031	0.076 ± 0.006[a)]
Malate+glutamate				
State 3	0.344 ± 0.069	0.180 ± 0.062[a)]	0.112 ± 0.037	0.069 ± 0.033
State 4	0.306 ± 0.101	0.191 ± 0.031	0.109 ± 0.035	0.061 ± 0.031[a)]

Results are expressed as nmol/min/mg protein. *a*) Significantly different ($p < 0.05$) from control group at each feeding period.

Table 4. Mitochondrial Antioxidant Enzyme Activities and Gluthatione Concentration in Rats Fed Control (C) and MSD

	3 mo		6 mo	
	C	MSD	C	MSD
T-GPX (U/mg)	145.2 ± 30.1	129.2 ± 33.6	114.2 ± 27.1	121.9 ± 17.8
Se-GPX (U/mg)	122.6 ± 21.1	109.5 ± 28.9	104.2 ± 24.4	109.7 ± 20.2
SOD (U/mg)	3.08 ± 0.99	3.46 ± 0.28	3.72 ± 0.62	3.09 ± 0.49
Glutathione (nmol/mg)	7.99 ± 2.79	7.36 ± 1.76	7.10 ± 0.48	8.85 ± 1.64[a)]

a) Significantly different ($p < 0.05$) from control group at each feeding period.

patic iron levels but decreased serum TIBC levels, which corresponded to serum transferrin levels (Figs. 2 and 3). Moreover, hepatic TfR levels were significantly elevated by MSD feeding (Fig. 4). From these data and our previous study,[11)] we assumed that IRP activation by oxidative stress (H_2O_2) might participate in the elevation of TfR by MSD feeding, resulting in iron accumulation in the liver.

Since mitochondria, especially the electron transport chain, are the major intracellular producers of O_2^- and H_2O_2,[26)] we focused on H_2O_2 production in mitochondria. Under normal physiological conditions, 1–5% of the oxygen consumed by mitochondria is converted to O_2^-, H_2O_2, and other ROS due to the "leakage" of unpaired electrons to molecular oxygen as they are being transported through the electron transport chain. If the respiratory chain is damaged for any reason such as hereditary factors, aging *etc*., electrons stop flowing through the chain, resulting in the elevation of mitochondrial ROS leakage, which might cause cellular damage. To our surprise, we found that excessive L-methionine consumption significantly decreased the rate of H_2O_2 generation from rat liver mitochondria, although it did not change mitochondrial O_2 consumption (Tables 2 and 3). Also, body weights and voluntary food of the rats fed MSD were significantly lower than those of rats fed the control diets (Fig. 1 and

data not shown). Gredilla *et al*.[27] and López-Torres *et al*.[28] demonstrated that short-term or long-term caloric restriction significantly decreased the H_2O_2 production of rat liver mitochondria without any alteration in oxygen consumption. They suggested that such caloric restriction might release less ROS per unit electron flow in the respiratory chain, consequently triggering a decrease in ROS production due to a decline in the degree of reduction of the complex I generator in the caloric-restricted animals. These data prompted us to speculate whether MSD feeding might decrease the reduction degree of electron carriers in the respiratory chain, resulting in a decrease in the H_2O_2 generation of mitochondria as seen in caloric restricted rats. Moreover, methionine is essential for the normal growth and development of mammals and is metabolized in the transmethylation-transsulfuration pathway. Excess methionine supplementation resulted in the hepatic accumulation of metnionine metabolite, such as S-adenosyl-L-homocystein, S-adenosyl-L-methionine, homocystein, cystathionine *etc*.[29] The rate of H_2O_2 generation is dependent upon at least 3 factors, namely, ambient oxygen concentration; amounts of autoxizable respiratory carriers such as NADH, $FADH_2$, and coenzyme Q; and the redox state of the autoxizable electron carriers.[10,30–32] The abnormal accumulation of such metabolites by excess methionine intake might cause liver dysfunction and then induce a redox state change in mitochondria, subsequently causing a decrease in H_2O_2 generation.

On the other hand, it has been reported that ethanol consumption decreased mitochondrial glutathione content and induced liver injury under various oxidative stresses. Such injury induced by ethanol treatment in rats was restored by S-adenosyl-L-methionine supplementation, which induced an enhancement of glutathione content.[33] In our study, MSD feeding significantly elevated glutathione concentration at 6 months, though it did not modify SOD or GPX activities (Table 4). These data indicated that MSD feeding might induce an accumulation of S-adenosyl-L-methionine in the liver and then an increase in glutathione concentration, ultimately resulting in the degradation of H_2O_2 in liver mitochondria.

Incidentally, the rate of H_2O_2 generation in control rats in our study was significantly lower at 6 months than at 3 months. According to the mitochondrial free radical theory of aging, mitochondrial ROS generation plays a casual role in the aging process.[34,35] Some findings suggest that mitochondrial oxygen consumption and the rate of ROS generation increase with aging.[36,37] The mechanism responsible for this increase is probably due to an enhancement of free radical leakage in the respiratory chain. It is well known that the maximum life span of rats is about 5 years. We investigated the H_2O_2 generation rate in the early part of the rat life span (5 months and 8 months of age in our study). In young rather than adult rats such as those under our experimental conditions, electrons are transferred through the chain smoothly, resulting in the release of less mitochondrial ROS, which might cause a reduction in H_2O_2 generation with aging.

As noticed above, the critical proteins that control the availability of iron within the cell are TfR and ferritin; their expression is modulated by the IRE-IRP regulatory system. In our present study, hepatic iron levels were higher but serum transferrin levels lower in MSD-fed rats than control rats. Furthermore, hepatic TfR levels significantly increased in MSD-fed animals compared with controls. These finding led us to speculate that excessive L-methionine intake may induce oxidative stress in the liver, and IRP1 activation by oxidative stress (H_2O_2) may participate in the elevation of TfR by MSD feeding. Nevertheless, we found that the resultant H_2O_2 production from mitochondria had significantly decreased. Whereas IRP1 activation by H_2O_2 has been extensively studied in cultured cells and *in vitro*, the physiological implications of IRP1 activation by H_2O_2 in intact organs have not yet received comparable attention. Mueller *et al*. demonstrated a novel regulatory connection between iron metabolism *via* IRP/IRE system and not only oxidative stress but also inflammation.[38] In our study, the neutrophils levels in the peripheral blood increased by MSD feeding (data not shown). Also, serum unsaturated iron-binding capacity (UIBC) and TIBC levels in MSD-fed rats were significantly lower than controls (serum iron levels tended to be lower in rats fed MSD). It is well known that the delocalization of intracellular iron is noticed in inflammation. These data suggested that increasing TfR by excess L-methionine consumption may be occurring in the inflammatory responses, as H_2O_2 is a main product of the oxidative reaction *in vivo*, rather than mitochondrial H_2O_2 generation.

In summary, excess L-methionine intake may stimulate TfR synthesis and subsequently result in iron accumulation in the liver. Conversely, MSD-feeding may affect mitochondrial function, resulting in a decrease in the H_2O_2 generation of rat liver

mitochondria, indicating that iron accumulation by MSD feeding may be responsible for another mechanism, *i.e.*, inflammatory response, rather than IRE-IRP system *via* oxidative stress (H_2O_2).

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